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# Phylogenetic Relationships in the *Festuca-Lolium* Complex (Loliinae; Poaceae): New Insights from Chloroplast Sequences

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The species within the *Lolium/Festuca* grass complex have dispersed and colonized large areas of temperate global grasslands both naturally and by human intervention. The species within this grass complex represent some of the most important grass species both for amenity and agricultural use worldwide. There has been renewed interest by grass breeders in producing hybrid combinations between these species and several countries now market *Festulolium* varieties as a combination of genes from both genera. The two genera have been differentiated by their inflorescence structure, but controversy has surrounded the taxonomic classification of the *Lolium-Festuca* complex species for several decades. In order to better understand the complexities within the *Lolium/Festuca* complex and their genetic background, the phylogeny of important exemplars from the *Lolium-Festuca* complex were reconstructed. In total 40 taxa representing the *Festuca* and *Lolium* species with *Vulpia myuros* and *Brachypodium distachyon* as outgroups were sampled, using two non-coding intergenic spacers (*trnQ-rps16*, *trnH-psbA*) and one coding gene (*rbcL*). Maximum parsimony (MP), Bayesian inference (BI) analyses based on each partition and combined plastid DNA dataset, and median-jointing network analysis were employed. The outcomes strongly suggested that the subgen. *Schedonorus* has a close relationship to *Lolium*, and it is also proposed to move the sect. *Leucopoa* from subgen. *Leucopoa* to Subgen. *Schedonorus* and to separate sect. *Breviaristatae* from the subgen. *Leucopoa*. We found that *F. californica* could be a lineage of hybrid origin because of its intermediate placement between the “broad-leaved” and “fine-leaved” clade.

**Keywords:** phylogeny, *Festuca*, *Lolium*, *trnQ-rps16*, *trnH-psbA*, *rbcL*

## INTRODUCTION

As one of the largest subtribes from the tribe Poeae (Pooideae, Poaceae), Loliinae encompasses nine genera (*Festuca*, *Lolium*, *Vulpia*, *Nardurus*, *Loliolum* Krecz, and Bobr, *Scleropoa*, *Cutandia* Willk., *Sphenopus*, and *Bellardiochloa* Chiov; Tzvelev, 1982; Soreng and Davis, 2000). Among the genera, *Festuca* is large and complex having more than 600 species with multiple ploidy levels ranging from diploid ( $2n = 2x = 14$ ) up to dodecaploid ( $2n = 12x = 84$ ) whereas *Lolium* is a small genus

with 10 recognized diploid species (Clayton and Renvoize, 1986; Loureiro et al., 2007). The two genera, *Festuca* and *Lolium* include a number of important grasses used as pasture, fodder, and amenity purposes. The *Lolium* genera include the two widely cultivated temperate grass species, *L. multiflorum* (annual or Italian ryegrass) and *L. perenne* (perennial ryegrass) which are characterized by rapid growth and high forage quality. There are more than 3000 cultivars grown around the world with many hybrids naturally or artificially produced (Cai et al., 2011). The *Festuca* genus also includes two agriculturally important forage crop species, tall fescue (*F. arundinacea*) and meadow fescue (*F. pratensis*). They differ from ryegrasses, having larger, deeper root systems and greater water and nutrient-use-efficiency, and generally higher stress tolerance than the ryegrasses. Another important group of *Festuca* species are the fine-leaved fescues which are valued for their forage, turf and ornamental use. Red fescue (*F. rubra* L.) and sheep fescue (*F. ovina* L.) are valued for their narrow leaves which minimize water loss and provide improved drought tolerance (Rognli et al., 2010).

The majority of species within the *Lolium/Festuca* grass complex are heterogeneous and largely obligate outbreeders; they are highly diverse in their growth ontogeny, morphology, and their adaptations to onsets of both climatic and edaphic stress. As a consequence they have dispersed and colonized large areas of temperate global grasslands (Humphreys et al., 2006). As a group, the *Festuca-Lolium* complex comprises species that are closely allied and are partially interfertile.

The two genera can be easily differentiated by their inflorescence structure, and the taxonomic classification of some *Lolium* and *Festuca* species are controversial. A number of taxonomic revisions have proposed placing the “broad-leaved” fescues (*Festuca* subgen. *Schedonorus*) into *Lolium* (Darbyshire, 1993) by natural phenomenon and through experimental evidence. This includes the regular occurrence of spontaneous hybridization between species of *Festuca* subgen. *Schedonorus* and chasmogamous species of *Lolium* which on the other hand only rarely occurs between the major “fine-leaved” fescues and *Lolium* (Stace, 1975; Barker and Stace, 1982). Furthermore, analysis of morphological data sets (Stebbins, 1956), DNA restriction site variation (Darbyshire and Warwick, 1992), and seed protein (Bulińska-Radomska and Lester, 1988) suggest placing *Lolium* and *Festuca* subgen. *Schedonorus* together as one lineage. In contrast, others have suggested that the broad leaf fescues be separated into a new genus called *Schedonorus* (Soreng and Terrell, 1997). Aside from the controversial relationship between “broad-leaved” fescues and *Lolium* species, the *Festuca* genus *per se* is also complex with the taxonomic placements of its specific subgenera, sections, and species quite intricate. For instance, sect. *Breviaristatae* was considered as separate from subgen. *Leucopoa* (Tzvelev, 1971; Clayton and Renvoize, 1986). However, Soreng et al. (1990) found that *F. slerophylla* of sect. *Leucopoa* had a close relationship with *F. arundinacea* of subgen. *Schedonorus* based on the chloroplast DNA restriction site variation. By the same method, Darbyshire and Warwick (1992) discovered that exemplars of sect. *Breviaristatae* had no phylogenetic affinity with exemplars of sect. *Leucopoa*, subgen. *Leucopoa*. Therefore, they suggested that sect. *Leucopoa* from

subgen. *Leucopoa* be moved to subgen. *Schedonorus*. *F. mairei* St. Yves, which is one of the key species in the evolution of polyploid fescues (Bulińska-Radomska and Lester, 1988) was first categorized into section *Scariosae* of subgen. *Festuca* (Stammers et al., 1995), but later was proposed to be reclassified into subgen. *Schedonrus* (Torrecilla and Catalán, 2002).

A better understanding of phylogenetic relationships within the *Festuca-Lolium* complex species would not only be very useful for future species conservation and for improved collection knowledge, but would also greatly assist future forage grass breeding programs. To ensure future grassland resilience and sustainable forage production for livestock agriculture, it has been considered as an increasingly important strategy to hybridize *Lolium* and *Festuca* species in order to gain and combine the complementary attributes of both. As *Lolium* x *Festuca* interspecific species' hybrids grass varieties are marketed under their own category termed *Festulolium* and provide a source of reliable, nutrient-use-efficient, and productive fodder for ruminants (Humphreys et al., 2014). Increased understanding of the phylogenetic relationships between the *Lolium/Festuca* species and of how the polyploid fescues and their adaptive benefits have evolved can benefit plant breeders and thereby accelerate the development of *Festulolium* breeding programs to better provide increased forage resilience sufficient to combat climate change.

Analysis of the phylogenetic relationships within the *Festuca-Lolium* complex encompassed the biological technology revolution from macro morphology to micro genetic level. Previous methods include chloroplast DNA (cpDNA) electrophoresis (Lehväslaiho et al., 1987; Soreng et al., 1990; Darbyshire and Warwick, 1992), RAPD (random amplification of polymorphic DNA) technology (Stammers et al., 1995; Wiesner et al., 1995), ITS (internal transcribe spacer) sequences of nuclear rDNA (Charmet et al., 1997; Gaut et al., 2000; Torrecilla and Catalán, 2002), and sequences of chloroplast *trnL-F* region (Catalán et al., 2004; Torrecilla et al., 2004). Despite the large genomic resources available for most intensively cultivated species of the *Festuca-Lolium* complex, conjoined analyses with chloroplast spacers as well as chloroplast genes have not been employed for the analysis of phylogeny among the *Festuca-Lolium* complex. The chloroplast has highly-conserved genes which are elementary to plants and are variable and informative regions over a long time scale. The use of cpDNA can also analyse the maternal source genome donor and has been applied successfully in the phylogenetic analysis of many taxa (Shaw et al., 2007; Sun, 2007; Nock et al., 2011).

In the present study, we sampled 42 taxa, including 28 *Festuca* taxa, 12 *Lolium* taxa and 2 related but out-group species (*Vulpia myuros*, *Brachypodium distachyon*). Chosen taxa have been identified by their morphology, they are representatives of broad-leaved and narrow-leaved species, these species are significant because of their importance in agricultural and amenity use and were therefore deemed the most important for this phylogenetic study. DNA sequence data from chloroplast spacers (*trnQ-rps16*, *trnH-psbA*) and chloroplast gene (*rbcL*) were used to resolve phylogenetic relationships among the *Festuca-Lolium* complex. The main objectives were to: (1) construct the plastid phylogeny

of *Festuca-Lolium* complex using two non-coding intergenic spacers and one coding gene, and compare with the previous analyses; (2) explore the maternal donors of the polyploid species of fescues.

## MATERIALS AND METHODS

### Taxon Sampling

A total of forty taxa were sampled from the *Lolium-Festuca* complex comprising 28 *Festuca* taxa corresponding to 3 subgenera, 7 sections, and one subsection, and 12 taxa of *Lolium*. *Vulpia myuros* and *Brachypodium distachyon* were included as out-groups based on previous phylogenetic studies of Loliinae (Inda et al., 2008). The taxa names, accessions numbers, ploidy level, origin and abbreviations are listed in **Table 1**. All the seed materials with PI were generously provided by the National Plant Germplasm System of USDA. The seeds were first germinated in petri dishes and then the strong seedlings were transferred to pots. Morphological observation and weeding were regularly undertaken in order to ensure the plant purity. Mitotic analyses of root tips were made to verify the ploidy level of each accession.

### DNA Extraction, Amplification, and Sequencing

Genomic DNA was extracted from freeze-dried leaf tissue of each accession by the standard CTAB (cetyl-trimethylammonium bromide) method (Doyle and Doyle, 1987) using the TIANcombi DNA PCR Kit (Beijing, China). One individual was sampled for DNA extraction, and five DNA samples were prepared for each taxon. The quality and concentration of the DNA were assessed by NanoVue Plus spectrophotometry produced by General Electric Company and checked on a 1% agarose-gel. The chloroplast *trnH-psbA* gene and *rbcL* were amplified with the universal primers (**Table 2**). The PCR (polymerase chain reaction) was performed in a final volume of 50  $\mu$ l, containing 4  $\mu$ l template DNA with the concentration of 10 ng/ $\mu$ l, 4  $\mu$ l primer with a concentration of 0.01 mmol/ $\mu$ l, 25  $\mu$ l 2 $\times$ Premix Taq (TaKaRa) with 0.4 mM dNTPs of each nucleotide, 3 mM MgCl<sub>2</sub> buffer, 1.25 U Taq DNA polymerase with pigment included, and addition of ddH<sub>2</sub>O to the final volume. The PCR amplification programs started with a 4 min initial denaturation step at 94°C; followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing (55°C for *rbcL* and 50°C for *trnH-psbA*), and 1.5 min extension at 72°C; ending with a final extension step at 72°C for 10 min. The PCR products were checked in a 1% agarose gel and purified with the AxyPrep DNA kit, and then qualified samples were sent to the Majorbio Company (Shanghai, China) for sequencing. Generally, 3–5 PCR products were sent for sequencing from each taxon.

### Data Analyses

The returned sequences data were initially spliced by the DNASTAR Seqman (Swindell and Plasterer, 1997) and aligned by the BioEdit ver. 7.0.9 (Hall, 1999). The nucleotide sites' information and the nucleotide frequencies were calculated by MEGA software ver. 5.02 (Tamura et al., 2011). The software package DAMBE ver. 5.5.29 (Xia and Xie, 2001) was used to

assess the substitution saturation by plotting pairwise rates of transitions and transversions against a correct genetic distance under F84 model.

In order to evaluate the divergence and relationship among taxa, number of sites (n), number of variable site (s), haplotype diversity (Hd) (Nei and Li, 1979), Tajuma's  $\pi$  (Tajima, 1989), Watterson's  $\theta_w$  (Watterson, 1975) were calculated, Neutrality test was also performed by the Tajima's and Fu and Li's D statistic (Tajima, 1989; Fu and Li, 1993). All the parameters above were conducted by DnaSP ver. 5.10 (Librado and Rozas, 2009).

### Phylogenetic Reconstruction

Phylogenetic analysis of each partition and the combined plastid DNA dataset (*trnQ-rps16*, *trnH-psbA*, *rbcL*) were created by maximum parsimony (MP) and Bayesian inference (BI). Maximum parsimony (MP) analyses were implemented in PAUP\* v.4.0b10 (Swofford, 2002). All characters were treated as unweighted and unordered, gaps were treated as "missing." The heuristic search option using the Tree Bisection-Reconnection (TBR) branch swapping and MUL-Tree option on, 10 replicates of random addition sequence with the stepwise addition option was employed to obtain the most parsimonious trees. The consensus tree option was set as "retain groups with frequency > 50%". Topological robustness MP analysis was evaluated by bootstrap analysis using a full heuristic search with 1000 replicates (Felsenstein, 1985) each with simple addition sequence.

Bayesian inference was carried out in MrBayes v.3.1.2 (Huelsenbeck and Ronquist, 2001). It performs Bayesian phylogenetic analysis of information from different data partition or the combined dataset. The optimal evolutionary model used for different data matrixes were estimated by jModelTest v.2.1.7 (Posada, 2008; Darriba et al., 2012) was used to determine the using the Akaike information criterion (AIC). The best-fit model was TPMluf+G for *trnQ-rps16* data, TIM1+I+G for *trnH-psbA* data, TIM2+G for *rbcL*, and TPMluf+I+G was chosen as the most appropriate for combined data analysis. Four MCMC (Markov Chain Monte Carlo) chain (one cold and three heated) were run for 200,000 generations for *trnQ-rps16* data, 600,000 generations for *trnH-psbA* data, and 120,000 generations for *rbcL* and combined data, each sampling every 10 generations. The analysis was continued until the standard deviation of split frequencies below 0.01. The first 5000, 15,000, and 3000 trees were stationary discarded as "burn-in" for *trnQ-rps16* data, *trnH-psbA* data and *rbcL* or combined data, respectively (determined empirically from the log-likelihood values using Tracer V1.4; Rambaut and Drummond, 2013). The remaining trees were employed to construct the 50%-majority rule consensus trees and frequencies of clades were evaluated by posterior probabilities (PP).

A Network representation may be more appropriate than the tree presentation when the existence of reticulate evolution such as gene transfer, hybridization, and recombination. The median-joining (MJ) network analysis can reveal the relationships between ancestral and derived haplotypes which was first employed to discuss the human mtDNA variation (Bandelt et al., 2000). Compared with other graph construction approaches

TABLE 1 | List of *Lolium/Festuca* examplers and the outgroup used in this study.

| Taxon  | Ploidy | PI        | Origin      | Abbr.  |
|--|--------|-----------|-------------|--------|
| <b>Festuca L.</b>                                  |        |           |             |        |
| <b>Subgen. Festuca</b>                             |        |           |             |        |
| <b>Sect. Festuca</b>                               |        |           |             |        |
| <b>Subject. Festuca ("F. ovina complex")</b>       |        |           |             |        |
| <i>Festuca hystrix</i>                             | 2x     | PI 302896 | Spain       | FHYS   |
| <i>Festuca idahoensis</i>                          | 4x     | PI 601053 | USA         | FIDA   |
| <i>Festuca ovina</i>                               | 2x     | PI 235218 | France      | FOVI   |
| <i>Festuca valesiaca</i>                           | 2x     | PI 634225 | Ukraine     | FVAL   |
| <i>Festuca brachyphylla</i>                        | 6x     | W6 25548  | Greenland   | FBRA   |
| <i>Festuca lemanii</i>                             | 6x     | PI 286207 | Czech       | FLEM   |
| <i>Festuca pseudovina</i>                          | 2x     | PI 374046 | Hungary     | FPSE   |
| <b>Sect. Aulaxyper Dumort ("F. rubra complex")</b> |        |           |             |        |
| <i>Festuca ampla</i>                               | 4x     | PI 283275 | Portugal    | FAMP   |
| <i>Festuca heterophylla</i>                        | 4x     | PI 249742 | Greece      | FHET   |
| <i>Festuca rubra</i>                               | 6x     | PI 595056 | Norway      | FRUB1  |
| <i>Festuca rubra</i>                               | 6x     | PI 318993 | Spain       | FRUB2  |
| <i>Festuca rubra subsp. Arctica</i>                | 6x,8x  | PI 659648 | Iceland     | FARC   |
| <b>Sect. Amphigenes (Janka) Tzvel</b>              |        |           |             |        |
| <i>Festuca pulchella</i>                           | 2x     | PI 287542 | Poland      | FPUL   |
| <b>Subgen. Schedonorus (P. Beauv.) Peterm.</b>     |        |           |             |        |
| <b>Sect. Plantynia (Dum.) Tzvelev</b>              |        |           |             |        |
| <i>Festuca gigantean</i>                           | 6x     | PI 206646 | Turkey      | FGIG   |
| <b>Sect. Schedonorus (P. Beauv.) Koch</b>          |        |           |             |        |
| <i>Festuca arundinacea</i>                         | 6x     | PI 634240 | France      | FARU   |
| <i>Festuca arundinacea subsp. atlantigena</i>      | 8x     | PI 577096 | UK          | FATL   |
| <i>Festuca arundinacea subsp. fenas</i>            | 4x     | PI 595048 | France      | FFEN   |
| <i>Festuca arundinacea subsp. orientalis</i>       | 6x     | PI 634282 | Ukraine     | FORI   |
| <i>Festuca pratensis subsp. pratensis</i>          | 2x     | PI 234777 | Germany     | FPRA   |
| <i>Festuca pratensis subsp. apennina</i>           | 4x     | PI 610808 | Switzerland | FAPE   |
| <i>Festuca mairei</i>                              | 4x     | PI 610941 | Morocco     | FMAI1  |
| <i>Festuca mairei</i>                              | 4x     | PI 283312 | Sweden      | FMAI2  |
| <b>Subgen. Leucopoa (Grised.) Hack</b>             |        |           |             |        |
| <b>Sect. Breviaristatae</b>                        |        |           |             |        |
| <i>Festuca altaica</i>                             | 4x     | PI 639774 | Mongolia    | FALT1  |
| <i>Festuca altaica</i>                             | 4x     | PI 236847 | Canada      | FALT2  |
| <i>Festuca californica</i>                         | 4x,8x  | W6 26789  | USA         | FCAL   |
| <b>Sect. Leucopoa</b>                              |        |           |             |        |
| <i>Festuca spectabilis</i>                         | 6x     | PI 383658 | Turkey      | FSPE1  |
| <i>Festuca spectabilis</i>                         | 6x     | PI 384871 | Iran        | FSPE2  |
| <i>Festuca kingii</i>                              | 8x     | PI 232305 | USA         | FKIN   |
| <b>Lolium. L</b>                                   |        |           |             |        |
| <i>Lolium multiflorum</i>                          | 2x     | PI 577241 | Italy       | LMUL1  |
| <i>Lolium multiflorum</i>                          | 2x     | PI 545668 | Turkey      | LMUL2  |
| <i>Lolium perenne</i>                              | 2x     | PI 547390 | Iran        | LPER1  |
| <i>Lolium perenne</i>                              | 2x     | PI 598510 | Turkey      | LPER2  |
| <i>Lolium rigidum</i>                              | 2x     | PI 254899 | Iraq        | LRIG1  |
| <i>Lolium rigidum</i>                              | 2x     | PI 516608 | Morocco     | LRIG2  |
| <i>Lolium temulentum</i>                           | 2x     | PI 422589 | Morocco     | LTEM1  |
| <i>Lolium temulentum</i>                           | 2x     | PI 298417 | Turkey      | LTEM2  |
| <i>Lolium persicum</i>                             | 2x     | PI 229764 | Iran        | LPERS1 |

(Continued)



TABLE 1 | Continued

| Taxon                          | Ploidy | PI        | Origin    | Abbr.  |
|--------------------------------|--------|-----------|-----------|--------|
| <i>Lolium persicum</i>         | 2x     | PI 545637 | Turkey    | LPERS2 |
| <i>Lolium subulatum</i>        | 2x     | PI 197310 | Argentina | LSUB   |
| <i>Lolium canariense</i>       | 2x     | PI 320544 | Spain     | LCAN   |
| <b>Out-group</b>               |        |           |           |        |
| <i>Vulpia myuros</i>           | 6x     | PI 204448 | Turkey    | VMYU   |
| <i>Brachypodium distachyon</i> | 2x     | W6 39443  | Turkey    | BDIS   |

TABLE 2 | Details of primer pairs used to amplify the *trnQ-rps16*, *trnH-psbA*, and *rbcL* gene.

| Region            | Primer name | Sequence (5'-3')              | References               |
|-------------------|-------------|-------------------------------|--------------------------|
| <i>trnQ-rps16</i> | trnQ        | GCG TGG CCA AGY GGT AAG GC    | Shaw et al., 2007        |
|                   | rps16       | GTT GCT TTY TAC CAC ATC GTT T |                          |
| <i>rbcL</i>       | 1F          | ATG TCA CCA CAA ACA GAA AC    | Kress and Erickson, 2007 |
|                   | 724R        | TCG CAT GTA CCT GCA GTA GC    |                          |
| <i>trnH-psbA</i>  | psbAF       | GTT ATG CAT GAA CGT AAT GCT C | Sang et al., 1997        |

(minimum-spanning network and statistical parsimony), the MJ network using the maximum parsimony (MP) method can provide the best measure of the true genealogy (Cassens et al., 2005). The MJ network was constructed by the Network 4.6.1.3 program (Fluxus Technology Ltd), the test of recombination was performed by the GARD Processor method with the HyPhy package (Pond and Muse, 2005). Based on the result of the test, the combination of the three plastid DNA datasets was used to generate the MJ network because of the absence of recombination signal in alignment (Log Likelihood = -4126.04; AIC = 8430.02).

## RESULTS

### Sequences Analyses

The length of *trnQ-rps16*, *trnH-psbA* and *rbcL* sequences were 700, 579, and 632 bp respectively, in the final aligned sequences of 40 taxa excluding the out-groups. The specific sites' information and nucleotide frequencies were shown in Table 3. Both the tests of substitution saturation for *trnQ-rps16*, *trnH-psbA* spacers and *rbcL* gene under the F84 model showed a basically linear regression which demonstrated no saturation effects among the mutation of different sequences (see Supplementary Figures 1–3).

The nucleotide diversity information containing the number of sites ( $n$ ), number of variable site ( $s$ ), haplotype diversity ( $H_d$ ), the average pairwise diversity ( $\pi$ ), and the diversity based on the number of segregating sites ( $\theta_w$ ) of *trnQ-rps16*, *trnH-psbA* spacers, and *rbcL* gene were calculated. The neutrality test results showed negative for the three sequences which might be because of the genetic bottleneck. Above values were displayed in Table 4.

All 126 sequences have been submitted to the database of NCBI (National Center for Biotechnology Information), the accession numbers are from KT43895 to KT439068 (see Supplementary Excel, Datasheet).

TABLE 3 | Features of the matched data matrix for *trnQ-rps16*, *trnH-psbA*, and *rbcL* gene sequences.

| Gene              | C   | V   | Pi | S  | ii  | si | sv |
|-------------------|-----|-----|----|----|-----|----|----|
| <i>TrnQ-rps16</i> | 557 | 106 | 68 | 38 | 540 | 12 | 13 |
| <i>trnH-psbA</i>  | 524 | 34  | 26 | 8  | 540 | 4  | 6  |
| <i>rbcL</i>       | 606 | 26  | 16 | 10 | 625 | 5  | 1  |

C, conserved sites; V, variable sites; Pi, parsimony-informative sites; S, singleton sites; ii, identical pairs; si, transitional pairs; sv, transversional pairs.

## Phylogenetic Analyses

### Chloroplast Spacer *trnQ-rps16* Data

The aligned *trnQ-rps16* sequences produced a total of 697 characters, of which 109 were variable and 75 were parsimony-informative. The parsimony analysis for *trnQ-rps16* sequences resulted in 215 most parsimonious trees (tree length = 215; consistency index = 0.9395; retention index = 0.9791; rescale consistency index = 0.9199). The 50% MP majority-rule consensus tree was identical to the tree obtained from BI except for some nodes presenting different statistical support. The tree shown in Figure 1 was MP tree with bootstrap support (BS) above the branches and posterior probabilities (PP) of BI tree below the branches. According to the tree, the two major clades had been strongly supported, mainly corresponding to the width of blades. The first clade included the narrow-leaved fescues and *Vulpia myuros*, the second clade contained all the *Lolium* samples and the broad-leaved *Festuca* taxa.

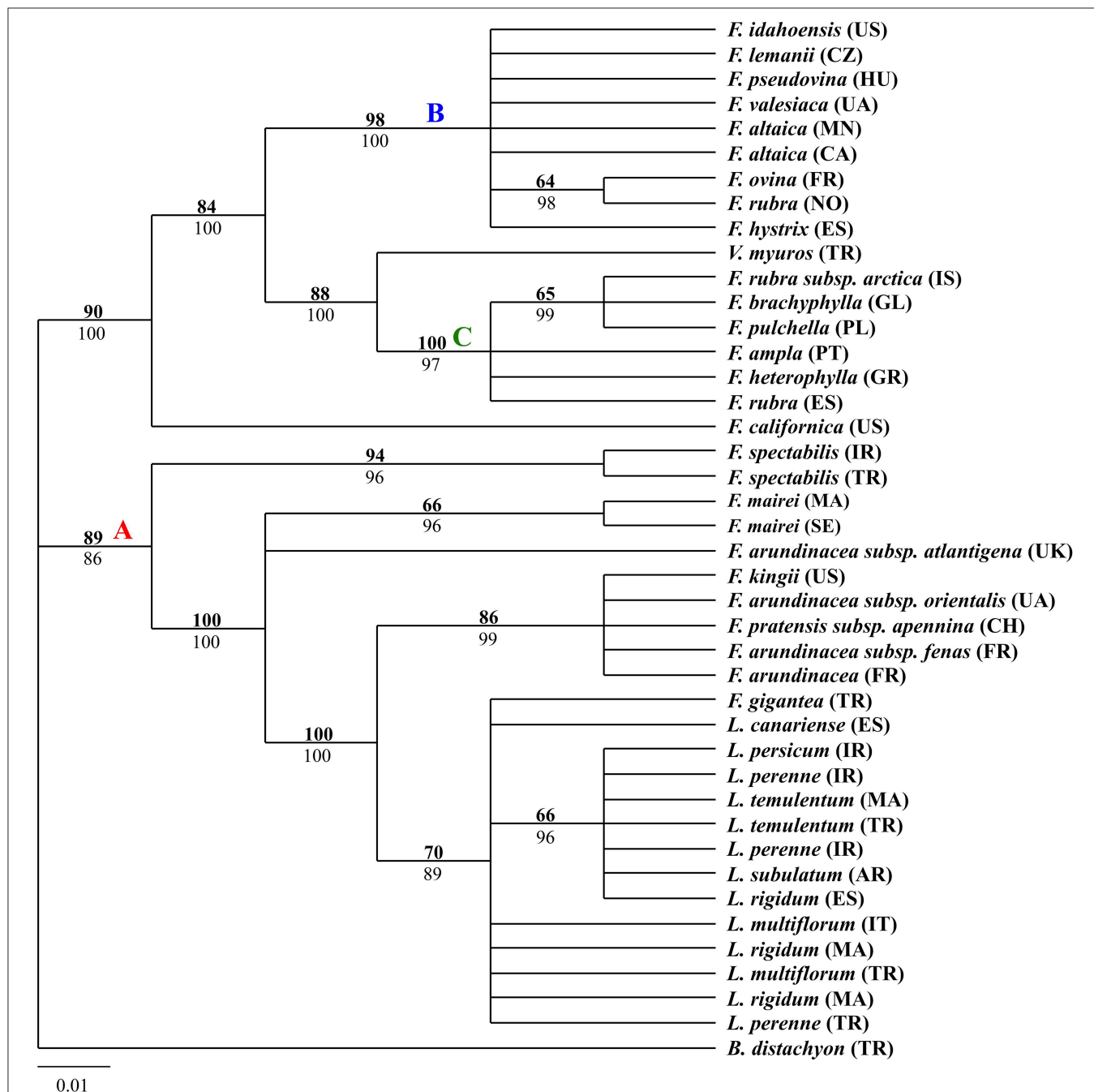
### Chloroplast Spacer *trnH-psbA* Data

The total character of the aligned *trnH-psbA* sequences was 585, of which 27 characters were variable and parsimony-informative. The parsimony analysis for *trnH-psbA* sequences resulted in 91 most parsimonious trees (tree length = 190; consistency index = 0.3000; retention index = 0.5994; rescale consistency index =

**TABLE 4 |** Estimates of nucleotide diversity and test statistics for *trnH-psbA*, *trnQ-rps16*, and *rbcL* gene sequences data sets.

| Gene              | <i>n</i> | <i>s</i> | $\Pi$   | Hd    | $\theta_w$ | Fu and Li's D           | Tajima's D             |
|-------------------|----------|----------|---------|-------|------------|-------------------------|------------------------|
| <i>trnQ-rps16</i> | 700      | 52       | 0.03736 | 0.871 | 0.03071    | −0.14073 ( $p > 0.10$ ) | 0.77642 ( $p > 0.10$ ) |
| <i>trnH-psbA</i>  | 579      | 31       | 0.01723 | 0.940 | 0.01337    | −0.12620 ( $P > 0.10$ ) | 0.99644 ( $P > 0.10$ ) |
| <i>rbcL</i>       | 632      | 26       | 0.01071 | 0.867 | 0.00967    | −1.03905 ( $p > 0.10$ ) | 0.36442 ( $p > 0.10$ ) |

*n*, total number of sites; *s*, number of polymorphic sites;  $\pi$ , nucleotide diversity per site; Hd, haplotype diversity;  $\theta_w$ , diversity based on the number of segregating sites.



0.1798). The 50% MP majority-rule consensus tree and the tree from BI were different but poorly resolved because of the limited difference among the sequences. The trees are shown in Supplementary Figure 4.

### Chloroplast Gene *rbcL* Data

The aligned *rbcL* sequences yielded a total of 632 characters with 26 variable characters and 16 informative characters among which the parsimony analysis for *trnH-psbA* sequences resulted in 48 most parsimonious trees (tree length = 51; consistency index = 0.8235; retention index = 0.9511; rescale consistency index = 0.7832). The 50% MP majority-rule consensus tree was highly congruent to the tree obtained from BI except for some nodes presenting different statistical support. The tree showed in **Figure 2** was MP tree with bootstrap support (BS) above the branches and posterior probabilities (PP) of BI tree below the branches. The tree outline was different from the other two trees. There were two major clades which had been well supported, one large clade including most of the taxa and one small clade including *F. ampla*, *F. rubra* subsp. *arctica*, *F. brachyphylla*, *F. heterophylla*, *F. pulchella*, and *F. rubra*.

### The Combined Dataset

Of 1926 total characters within the combined data set of the three plastid DNA regions, 135 characters were variable, and 128 characters were informative. The cladistics parsimony search yielded 363 most parsimonious trees with the tree length of 368 steps, a consistency index (CI) of 0.7853, retention index (RI) of 0.9349, and rescale consistency index (RC) of 0.7342. The 50% MP majority-rule consensus tree was largely incongruent to the tree obtained from BI analyses (**Figure 3**). According to the classification, the 50% MP tree was mainly influenced by the sequences of the *rbcL* and the BI tree was largely affected by the *trnQ-rps16* sequences data. The two major clades were highly supported in the BI tree rather than in the MP tree.

### Network Analysis

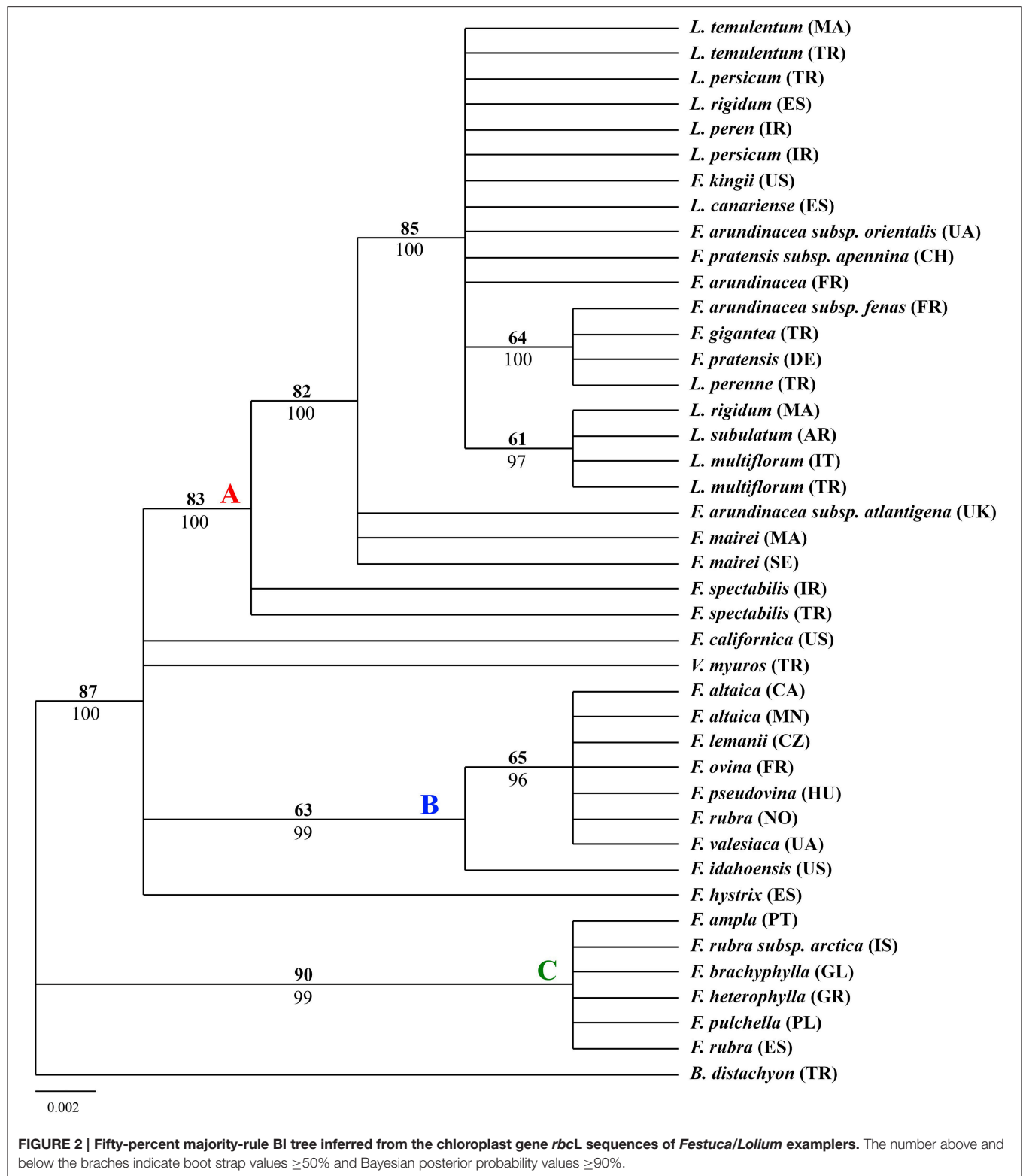
In order to get better insights into the number of haplotypes of the combined sequences and their relations, a median-joint network was employed. Each circular network node represents a single sequence haplotype, with node size being proportional to the number of isolates with the haplotype. Mv (median vectors representing missing intermediates) reveals unsampled nodes inferred by MJ network analysis, and the number along the branches shows the number of mutations. 33 haplotypes were derived from 40 taxa which revealed higher levels of haplotype diversity of the combined sequence data (**Figure 4**). MJ analysis generally grouped according to the clades shown by the phylogenies of the combined data. The taxa were grouped into three clades, and the clade I and clade II could be considered as one group, and clade I and II were 46 and 35 mutational steps from clade III, respectively. All the *Lolium* taxa were nested with all the broad-leaved *Festuca* taxa.

## DISCUSSION

From the combined analyses of the chloroplast spacers *trnQ-rps16*, *trnH-psbA*, the chloroplast gene *rbcL*, and the combined data set and the MJ network of the combined data, the dendrograms achieved all clearly demonstrate three obvious clades. The largest clade (A) contained all the *Lolium* taxa and included the broad-leaved fescues including all the samples of subgenus *Schedonorus* (*F. arundinacea*, *F. pratensis*, *F. mairei* and *F. gigantea*) and samples from sect. *Leucopoa* of Subgenus *Leucopoa* (*F. spectabilis* *F. kingii*). A smaller clade (B) included all the samples from subsect. *Festuca* including *F. valesiaca*, *F. ovina*, *F. hystrix*, *F. lemanii*, *F. pseudovina*, *F. idahoensis* as well as *F. altaica* and *F. rubra* (NO). The only species of subsect. *Festuca* not included within this clade is *F. brachyphylla*. A smaller third clade (C) included samples from sect. *Aulaxyper* and sect. *Amphigenes* of subgenus *Festuca* (*F. ampla*, *F. heterophylla*, *F. rubra* (ES), *F. rubra* subsp. *arctica*, *F. pulchella*) and *F. brachyphylla*. In the dendrogram of *trnQ-rps16* sequences and BI tree of the combined data set, clade B and C with *F. californica* formed one clade, while in the dendrogram of *rbcL* sequences and MP tree of the combined data set, a new clade was made up by clade A, clade B, and *F. californica*.

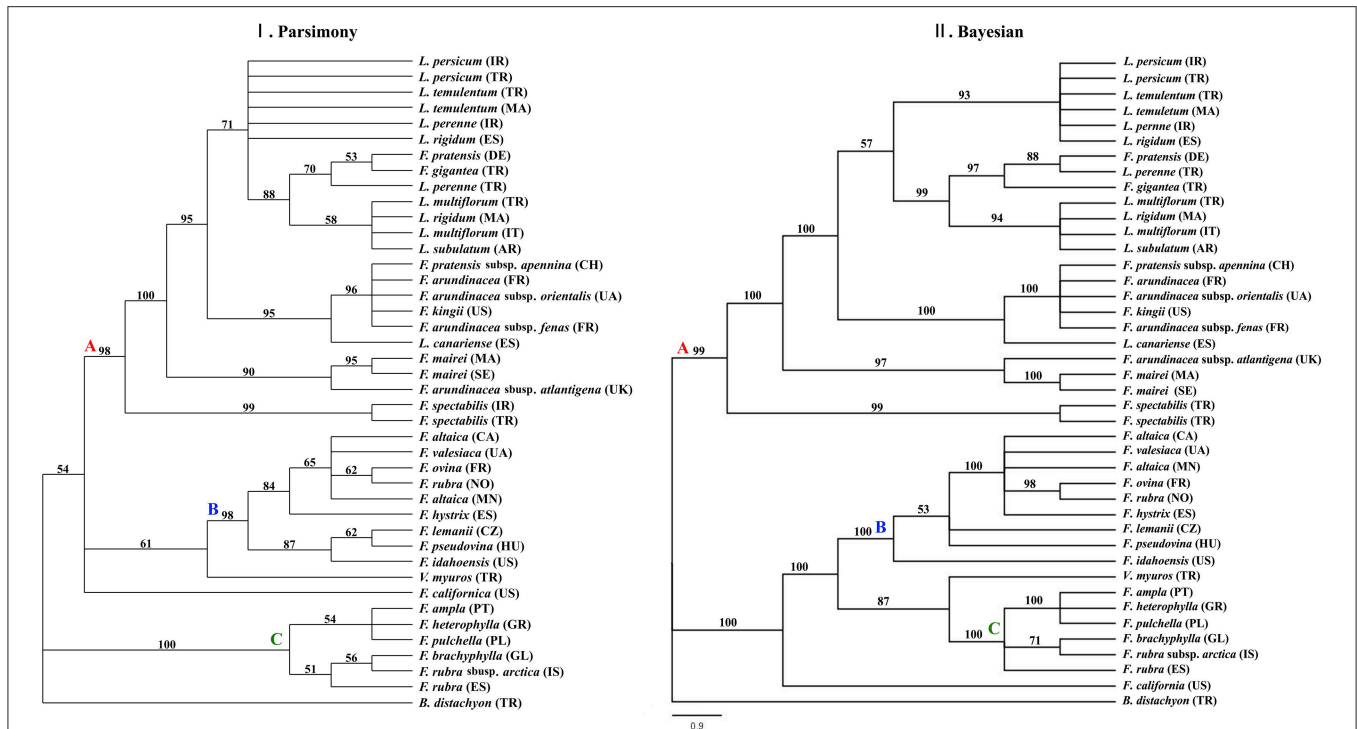
As all the *Lolium* species were nested closely with the broad-leaved *Festuca* species in clade A (**Figures 1–4**), it could be concluded that the broad-leaved fescues have closer relationship to *Lolium* grass species than to the fine-leaved fescues. There has been debate about the classification of subgen. *Schedonorus*. It was suggested that the subgen. *Schedonorus* (broad-leaved fescues) be included within *Lolium* (Darbyshire, 1993) despite the obvious differences in their inflorescence morphology (raceme for *Festuca* and spica for *Lolium*), whilst others have suggested a split of the subgen. *Schedonorus* into an independent genus, *Schedonorus* (Soreng and Terrell, 1997). According to the result demonstrated within the current study, the former classification seems more reasonable, in other words, subgen. *Schedonorus* has a close relationship to *Lolium*. Among all the exemplars of *Lolium*, only *L. canariense*, which is found mainly on poor land in maritime condition (Loos, 1994), has a closer relationship to broad-leaved fescues than to other *Lolium* species. Two representatives of sect. *Leucopoa* (*F. kingii* and *F. spectabilis*) were placed in the clade A with representatives from subgen. *Schedonorus* and *Lolium*, while for the representatives of Sect. *Breviaristatae*, *F. altaica* taxa were attached to clade B and *F. californica* has developed as an individual group. Similar results were achieved previously where the phylogenetic relationships among the *Festuca-Lolium* complex were described using SRAP markers (Cheng et al., 2015) and in earlier studies (Catalán et al., 2004, 2007; Inda et al., 2008). From the current and previous research, we strongly propose that the sect. *Leucopoa* should be moved from subgen. *Leucopoa* to Subgen. *Schedonorus* or into a separate sect. *Breviaristatae* from the subgen. *Leucopoa*. According to the strict consensus tree and Bayesian 50% MR consensus tree inferred from ITS and *trnL-F* sequences, representatives of sect. *Breviaristatae* (*F. altaica* and *F. californica*) have an intermediate placement between the “broad-leaved” and “fine-leaved” clade (Catalán et al., 2004). In





the current study, the lineage of *F. californica* could indicate a hybrid origin due to its intermediate placement between the “broad-leaved” (A) and “fine-leaved” clade (B).

Cytological investigations at the Institute for Biological, Environmental, and Rural Sciences (IBERS) using genomic *in situ* hybridization (GISH) and as total genomic DNA probes,



**FIGURE 3 | Phylogenies inferred from the combined dataset of *Festuca/Lolium* exemplars using maximum parsimony and Bayesian inference.**

Fifty-percent majority-rule BI tree inferred from the combined dataset of *Festuca/Lolium* exemplars. (I) The MP majority rule consensus tree with the numbers at nodes indicating bootstrap values  $\geq 50\%$ . (II) The 50% majority rule consensus tree from the Bayesian inference with the numbers at nodes indicating Bayesian posterior probability values  $\geq 90\%$ .

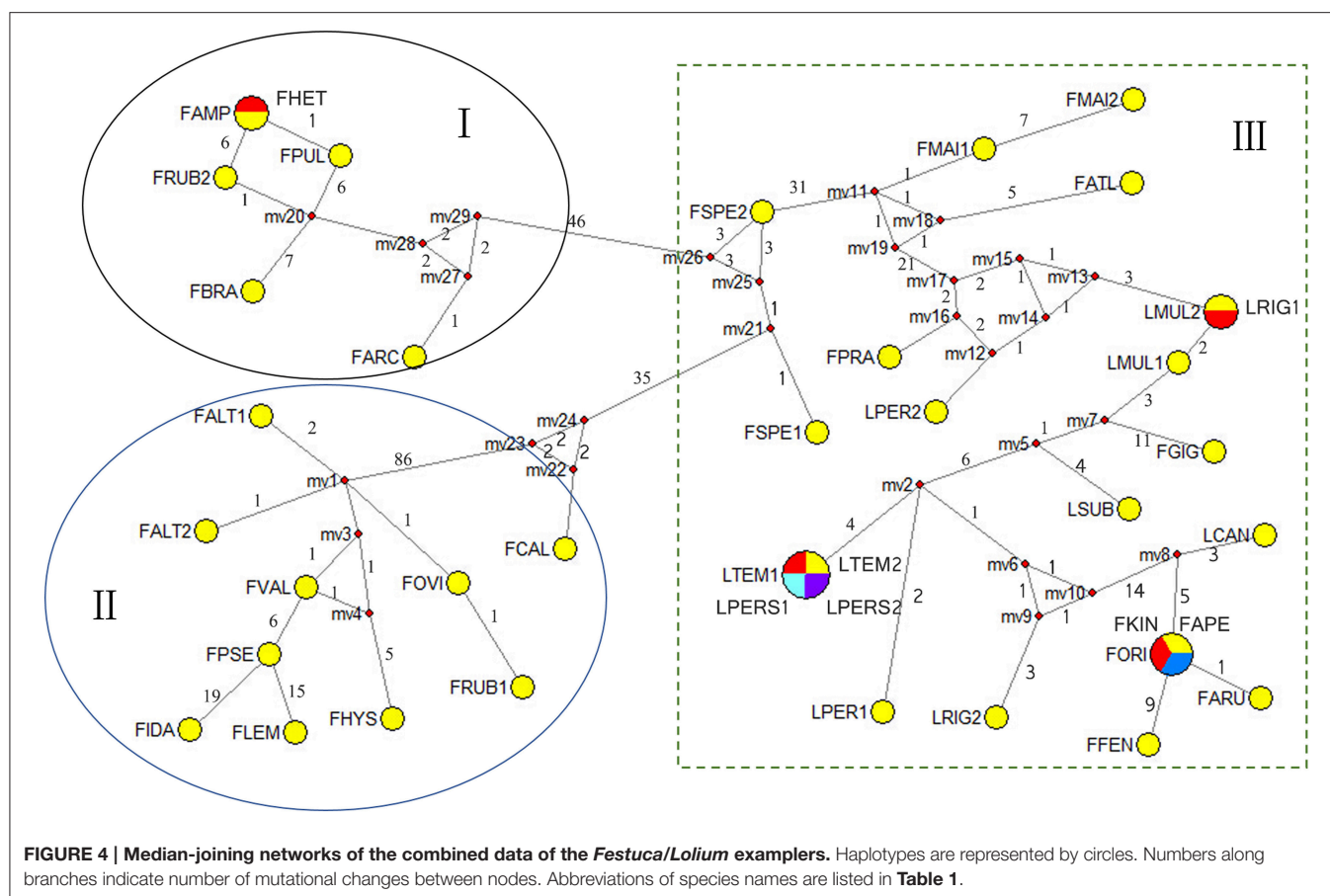
candidate *Festuca* species have established the close ancestry of *F. pratensis*, *F. arundinacea* var. *glaucescens* (also known as *F. arundinacea* subsp. *fenas*) and *F. arundinacea* (Humphreys et al., 1995). From all the dendrograms in this study, *F. arundinacea*, *F. arundinacea* subsp. *fenas*, *F. pratensis* subsp. *apennina*, *F. arundinacea* subsp. *orientalis*, and *F. kingii* were all gathered closely within one group and thus might share the same ancestry. In the MJ network, *F. pratensis* subsp. *apennina*, *F. arundinacea* subsp. *orientalis*, and *F. kingii* shared the same haplotype, and they are also closely associated to *F. arundinacea*, *F. arundinacea* subsp. *fenas*. The North African fescue species *F. mairei* and *F. arundinacea* subsp. *atlantigena* were classified into one group which showed their close relationship. *F. mairei* was once been placed in sect. *Scariosae* of subgen. *Festuca* using a RAPD analysis (Stammers et al., 1995). However, based on the previous SRAP analysis (Cheng et al., 2015) and the research presented in this paper, it is considered as more accurate to place *F. mairei* within the subgen. *Schedonorus*.

In all clade B studies herein, *F. brachyphylla* was separated from other exemplars of the Subsect. *Festuca* of Subgen. *Festuca* and was associated closer to representatives of Sect. *Aulaxyper* and Sect. *Amphigenes*. *F. brachyphylla* used to be considered as an arctic-alpine counterpart to the more temperate-montane *F. ovina*, which both belong to section *Festuca*, but the delimitation of *F. brachyphylla* and *F. ovina* has been considered as controversial with some authors having concluded the taxa

of *F. brachyphylla* as a subspecies of *F. ovina* (Cronquist et al., 1977) whilst other authors have included both taxa in a widely defined *F. brachyphylla* (Ejellheim et al., 2001). In the present study, *F. brachyphylla* was differentiated from *F. ovina*, as shown previously by the same authors using SRAPs markers (Cheng et al., 2015) and also from a Bayesian tree of *Loliinae* which used *trnT* and ITS data (Inda et al., 2008). It is proposed that the *F. brachyphylla* taxa be considered as a separate entity form Subsect. *Festuca*. In addition, *F. altaica* of sect. *Breviaristatae* was found closely aligned to subsect. *Festuca* known as the “*F. ovina* complex.”

In clade C, *F. pulchella* of Sect. *Amphigenes* had a close relationship with sect. *Aulaxyper* known as “*F. rubra* complex.” Two exemplars of *F. rubra* were clustered within two different groups, it might be because of the large latitude difference. From the MJ network, *F. ampla* shared the same haplotype with *F. heterophylla* which indicated a close relationship between them.

In conclusion, as the two major genera of the grass family, the *Lolium* and *Festuca* taxa can be considered to have expanded to become the predominant temperate grassland of the world. From a taxonomical perspective, it is essential to classify the different species into their correct sections or subsections of subgenera as well as to clarify the relationships of some important species. From an agricultural perspective, it has become increasingly important to hybridize *Lolium* and *Festuca* species in order to gain the attributes of both. Current synthetic *Festulolium* hybrids



are frequently genetically unstable, and so it is important to understand how stable polyploids within the *Festuca* taxa have evolved from their progenitor species. In the present work, from the analyses of three plastid DNA data, either from the MP trees or BI trees, from the single or combined data, it is shown that the subgen. *Schedonorus* shares a close relationship with the majority of *Lolium* grasses. The phylogenetic tree can guide the parents chosen for hybrid breeding. It is recommended that *F. mairei* should be included within the subgen. *Schedonorus*. *F. californica* could have a lineage of hybrid origin because of its intermediate placement between the “broad-leaved” and “fine-leaved” clades. Furthermore, it is suggested that *F. brachyphylla* should be treated as a separate entity from the “*F. ovina* complex.” The results add more information and understanding into species evolution within the *Lolium/Festuca* complex.

## AUTHOR CONTRIBUTIONS

YC performed the experiments, analyzed the data and wrote the manuscript. KZ and HY guided the bioinformatics analyses, XZ

organized the funding and participated in the samples collecting, XM guided the manuscript writing, JH, and MH provided helpful comments and language editing on the manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <http://journal.frontiersin.org/article/10.3389/fevo.2016.00089>

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**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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